Epithelial Mesenchymal Transition by c-Fos Estrogen Receptor Activation Involves Nuclear Translocation of β-Catenin and Upregulation of β-Catenin/Lymphoid Enhancer Binding Factor-1 Transcriptional Activity

Andreas Eger,* Andreas Stockinger,* Birgit Schaffhauser,* Hartmut Beug,‡ and Roland Foisner*

*Department of Biochemistry and Molecular Cell Biology, University of Vienna, A-1030 Vienna, Austria; and ‡Research Institute of Molecular Pathology, A-1030 Vienna, Austria

Abstract. Mouse mammary epithelial cells expressing a fusion protein of c-Fos and the estrogen receptor (FosER) formed highly polarized epithelial cell sheets in the absence of estradiol. β-Catenin and $p120^{ctn}$ were exclusively located at the lateral plasma membrane in a tight complex with the adherens junction protein, E-cadherin. Upon activation of FosER by estradiol addition, cells lost epithelial polarity within two days, giving rise to a uniform distribution of junctional proteins along the entire plasma membrane. Most of the β-catenin and $p120^{ctn}$ remained in a complex with E-cadherin at the membrane, but a minor fraction of uncomplexed cytoplasmic β-catenin increased significantly. The epithelial–mesenchymal cell conversion induced by prolonged estradiol treatment was accompanied by a complete loss of E-cadherin expression, a 70% reduction in β-catenin protein level, and a change in the expression pattern of $p120^{ctn}$ isoforms. In these mesenchymal cells, β-catenin and $p120^{ctn}$ were localized in the cytoplasm and in defined intranuclear structures. Furthermore, β-catenin colocalized with transcription factor LEF-1 in the nucleus, and coprecipitated with LEF-1–related proteins from cell extracts. Accordingly, β-catenin–dependent reporter activity was upregulated in mesenchymal cells and could be reduced by transient expression of exogenous E-cadherin. Thus, epithelial mesenchymal conversion in FosER cells may involve β-catenin signaling.

Key words: cell adhesion • E-cadherin • junctional complexes • polarized epithelium • wnt signaling

Introduction

In monolayers formed by fully polarized epithelial cells, tight adhesion is mediated by specialized adhesive junctions at their lateral plasma membranes, including actin filament-associated adherens junctions and intermediate filament-associated desmosomes. Adherens junctions are specified by the transmembrane glycoprotein E-cadherin, whose extracellular domain mediates calcium-dependent, homophilic cell–cell adhesion between adjacent cells (Shapiro et al., 1995; Nagar et al., 1996; Tomschy et al., 1996; Yap et al., 1997; Adams et al., 1998). The intracellular domain of E-cadherin associates with a group of proteins, collectively termed catenins (Nathke et al., 1994). β-catenin and plakoglobin (γ-catenin) interact directly with the cytoplasmic domain of cadherins in a mutually exclusive way (Hinck et al., 1994; Aberle et al., 1996). They belong to the armadillo protein family characterized by 13 consecutive 42 amino acid repeats in the middle of the polypeptide, which are involved in the interaction with E-cadherin (for review see Cowin and Burke, 1996). At their NH_2 termini, both β-catenin and plakoglobin bind to the vinculin-related protein, α-catenin, which, in turn, links the cadherin/catenin complex to the actin cytoskeleton, either directly or via interaction with α-actinin (reviewed in Cowin and Burke, 1996) and vinculin (Weiss et al., 1998). A protein originally identified as a substrate for src tyrosine kinase, $p120^{ctn}$, was later identified as a member of the catenin protein family (for review see Daniel and Reynolds, 1997) and was found to interact with the cytoplasmic juxtamembrane portion of E-cadherin, a region involved in the regulation of cadherin clustering and cell adhesion (Ozawa and Kemler, 1998; Yap et al., 1998). Both cadherin clustering (Adams et al., 1998; Yap et al., 1997) and the interaction of the cadherin/catenin complex with the actin cytoskeleton (Nagafuchi et al., 1994; Aberle et al., 1996) have been described as crucial for the formation of stable cell–cell contacts, but the detailed molecular mechanisms are still not completely understood.

Address correspondence to Dr. Roland Foisner, Department of Biochemistry and Molecular Cell Biology, Biocenter, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria. Tel.: 43-1-4277-52856. Fax: 43-1-4277-52854. E-mail: foisner@abc.univie.ac.at

In the past years, it became clear that cadherin-mediated adhesion is a highly dynamic process that is regulated by several signal transduction pathways (Eger and Foisner, 2000). For instance, activation of various tyrosine kinase-dependent pathways has been described to lead to phosphorylation of β-catenin, plakoglobin, and $p120^{ctn}$, and to downregulate E-cadherin–based cell adhesion (for reviews see Barth et al., 1997; Daniel and Reynolds, 1997). In addition, β-catenin has been identified as a direct component of the wnt/wingless signaling cascade (for recent reviews see Barth et al., 1997; Ben-Ze'ev and Geiger, 1998; Willert and Nusse, 1998). This pathway is highly conserved from flies to mammals and plays a central role in the determination of cell fate decisions during embryonic development (Huber et al., 1996a; Moon et al., 1997; Willert and Nusse, 1998). In the absence of wnt signaling, cytosolic β-catenin was found in a complex with the adenomatous polyposis coli (APC)[1] tumor suppressor protein and axin (or the related protein conductin; Rubinfeld et al., 1996; Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998) and was described to be constitutively phosphorylated by GSK3β on NH_2-terminal serine residues (Yost et al., 1996) that targets the protein for ubiquitination and subsequent proteosomal degradation (Aberle et al., 1997; Orford et al., 1997). Inhibition of GSK3β upon wnt signaling leads to the stabilization and accumulation of β-catenin in the cytoplasm and in the nucleus, where it forms a complex with transcription factors of the T cell factor/lymphoid enhancer-binding factor-1 (TCF/LEF-1) family and regulates the expression of various target genes (Behrens et al., 1996; Huber et al., 1996b; Molenaar et al., 1996).

Several observations have implicated β-catenin in carcinogenesis. These include the phosphorylation of β-catenin by oncogenic versions of tyrosine kinases (Barth et al., 1997; Daniel and Reynolds, 1997), the interaction of β-catenin with the tumor suppressor protein APC (Barth et al., 1997; Daniel and Reynolds, 1997; Gumbiner, 1997) and its identification as a downstream target of the oncogene Wnt-1. Moreover, mutations in β-catenin, increased protein levels of β-catenin, and/or a constitutive transcriptional activity of β-catenin/TCF complexes have been demonstrated in various colon and melanoma cancer cell lines and in hepatocellular carcinomas (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997; de La Coste et al., 1998). The oncogene c-myc has been identified as a potential target gene for β-catenin/TCF-4 transcriptional activity in colon cancer cell lines (He et al., 1998). Also, the ectopic expression of a stabilized truncated β-catenin in keratinocytes of transgenic mice resulted in the development of hair tumors (Gat et al., 1998). Cancer progression has also often been linked to an abnormal down-regulation or even loss of cadherins, leading to an increase in the invasiveness of primary tumor cells (Perl et al., 1998). A direct link between β-catenin's signaling function and E-cadherin expression levels in cancer cells, however, has not been established yet. Putting all available data together, it is likely that the development of cancer in epithelial tissues on the one hand, and epithelial–mesenchymal transition (EMT) during embryogenesis on the other, both utilize the same basic mechanisms that regulate the function(s) of cadherins and catenins in cell adhesion and signaling.

In the present study, we investigated the disintegration of junctional complexes after loss of epithelial polarity and EMT, and the diverse functions of β-catenin in cell adhesion and signaling. For this purpose, we used a previously described murine mammary epithelial cell line (IM-Ep-1-FosER; Reichmann et al., 1992). FosER cells constitutively express a fusion protein consisting of the c-fos oncogene and the hormone-binding domain of the human estrogen receptor, whose transactivating function is regulated by the presence of estradiol in the medium. In the absence of estradiol, FosER is inactive and cells form highly polarized epithelial cell sheets, whereas addition of estradiol to the culture medium was shown to induce FosER activity and to cause loss of epithelial polarity and EMT characterized by loss of E-cadherin and upregulation of mesenchymal markers (vimentin, proteases, and protease inhibitors). Here, we show that the majority of β-catenin persisted in a complex with E-cadherin during loss of epithelial polarity caused by short-term activation of FosER. However, a small fraction of β-catenin also accumulated in an E-cadherin–independent, cytoplasmic pool. After EMT induced by prolonged FosER activation, loss of E cadherin was accompanied by translocation of a significant fraction of cellular β-catenin to nuclear sites, frequently colocalizing with LEF-1. Concomitantly, LEF-1–dependent reporter gene activity was increased in these mesenchymal cells. Our data provide evidence that β-catenin/LEF-1 signaling is activated upon FosER-induced EMT.

Materials and Methods

Cell Culture

Mouse mammary epithelial cells constitutively expressing c-Fos estrogen receptor fusion proteins (FosER, Reichmann et al., 1992) or c-Jun estrogen receptor fusion proteins (JunER, Fialka et al., 1996) were cultured on 24-mm Falcon (Becton Dickinson) or 75-mm Transwell (Corning Costar) permeable filter inserts in high glucose DME, supplemented with 8% FCS, 10 mM Hepes, pH 7.2, 50 U/ml penicillin, and 50 μg/ml streptomycin (all Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2. To activate the FosER fusion protein and induce loss of polarity, cells were cultured in DME containing 1 μM β-estradiol (Sigma-Aldrich Chemie GmbH). Mesenchymal FosER cells were obtained by an initial treatment of polarized FosER cells with estradiol for 3–4 d, followed by several passages in the presence of estradiol for 2–3 wk.

Metabolic Labeling and β-Catenin Turnover Analysis

Epithelial FosER cells, grown on filters, 4-d estradiol-treated, and mesenchymal FosER cells were incubated in methionine-free DME medium containing 100 μCi/ml L-[^{35}S]methionine (New England Nuclear Life Science Products, Inc.) for 90 min (pulse), and chased in complete medium for 1–7 h. β-Catenin was immunoprecipitated from cell lysates in buffer containing 1% Triton X-100 and 0.1% SDS (Eger et al., 1997), using mAb to β-catenin (see below) and protein G–Sepharose (Sigma Chemical Co.). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Radioactive label in β-catenin bands was determined by scintillation counting and the amount of protein was analyzed by densitometric scanning of Coomassie-stained gel bands.

[1]*Abbreviations used in this paper:* APC, adenomatous polyposis coli; EMT, epithelial mesenchymal transition; FosER, c-Fos estrogen receptor; HA, hemagglutinin; LEF, lymphoid enhancer binding factor; TCF, T cell factor.

Transient Transfections and Reporter Gene Assays

Transient transfection experiments were performed in 60-mm plastic dishes (Nunc) using lipofectamine (Life Technologies) according to the manufacturer's instructions. The following were used: 2 μg luciferase reporter plasmids (pTopflash or pFopflash; Korinek et al., 1997) and 2 μg β-galactosidase control reporter plasmid (pAD-CMV1:βgal, kindly provided by D. Gründler-Thompson, Boehringer-Ingelheim, Vienna, Austria); 1 μg pTopflash/pFopflash, 1 μg pAD-CMV1:βgal, and 5–10 μg E-cadherin expression vector (pMexE-cadherin) or empty vector (pMexNeo, both provided by G. Christofori, IMP, Vienna, Austria); 1 μg pTopflash/pFopflash, 1 μg pAD-CMV1-βgal, and 5 μg β-catenin expression vector (pCS2+mtMMBC6*myc) or empty vector (pCS2+6*myc, both kindly provided by R. Kemler, Max-Planck Institute of Immunobiology, Freiburg, Germany; Aberle et al., 1997); 1 μg pTopflash/pFopflash, 1 μg pAD-CMV1-βgal, and 5 μg mutated β-catenin expression vector (pCS2MMBCS33A6*myc) or empty vector (pCS26*myc, Aberle et al., 1997); or 1 μg pTopflash/pFopflash, 1 μg pAD-CMV1:βgal and 1 μg hemagglutinin (HA)-tagged mouse LEF-1 expression vector (Giese and Grosschedl, 1993) or empty vector. After 48 h, cells were lysed in 200 μl 0.25 M Tris/HCl, pH 7.5, 0.5% Triton X-100, and soluble fractions were used for β-galactosidase and luciferase assays according to standard protocols.

Antibodies

The following immunoreagents were used: multiepitope cocktail to desmoplakin I and II (Progen Biotech.); mAbs to β-catenin, plakoglobin, $p120^{ctn}$, and E-cadherin (Transduction Labs); mAb to ZO-1 (Chemicon Intl., Inc.); antiserum to β-catenin and mAb to actin (Sigma Chemical Co.); antiserum against APC (kindly provided by I.S. Näthke and W.J. Nelson, Stanford University School of Medicine, Stanford, CA); and affinity-purified antiserum M16C against an NH_2-terminal region of mouse LEF-1 (kindly provided by O. Huber and R. Kemler; Aberle et al., 1997). Secondary antibodies coupled to peroxidase or alkaline phosphatase were obtained from Promega or BioRad. Secondary antibodies conjugated to Bodipy FL were purchased from Molecular Probes, Inc., antibodies coupled to Texas red were from Accurate Chemical and Scientific Corp., or from Jackson ImmunoResearch Labs, and goat anti–mouse secondary antibodies coupled to 10-nm gold particles and goat anti–rabbit secondary antibodies coupled to 5-nm gold particles were from BioCell Research Lab.

Immunofluorescence and Electron Microscopy

For immunofluorescence microscopy, FosER cells were fixed on filters in methanol/acetone (1:1) for 3 min at −20°C. Alternatively, cells or cryosections (see below) were fixed with 2.5% paraformaldehyde in PBS for 30 min at 20°C, permeabilized in 0.2% Triton X-100 for 15 min, and incubated in 50 mM NH_4Cl/0.01% glycine. After fixation, filters were cut in small pieces, incubated in 0.2% gelatin or 10% FCS/0.01% glycine for 30 min, and stained with the primary and secondary antibodies for 60 min each at 20°C. For DNA staining, samples were subsequently treated with 10 μg/ml RNase A (Boehringer Mannheim, Corp.) and stained with 0.1 μg/ml propidium iodide (Sigma Chemical Co.). Samples were mounted in Mowiol and viewed in a Zeiss Axiophot microscope and an MRC 600 confocal microscope (BioRad).

For ultrastructural analyses, cells were fixed in 0.5% glutaraldehyde in 0.25 M Hepes buffer, pH 7.4, for 1 h at 20°C and in 1% osmium tetroxide in PBS for 1 h at 4°C, washed three times in PBS and dehydrated in a graded series of ethanol (30, 50, 70, and 90% for 15 min each, and 100% twice for 30 min). Samples were incubated in propylenoxid/Agar 100 (1:1; Agar Scientific Ltd.), in propylenoxid/Agar 100 (1:2) for 90 min each, and in Agar 100 for 18 h, with changes of embedding medium after 2 and 16 h. Subsequently, the epoxy resign was polymerized at 60°C for 3 d. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome and stained with uranyl acetate and lead citrate.

For cryosectioning, cells were fixed with 4% paraformaldehyde in 250 mM Hepes, pH 7.4, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$ for 1 h at 20°C, embedded in 10% gelatine, postfixed in 0.5% glutaraldehyde for 30 min, incubated in 2.3 M sucrose in PBS for 16 h, and frozen in liquid nitrogen. Cryosectioning and immunolabeling were done as described in Eger et al. (1997) using rabbit antiserum to β-catenin, mAb to $p120^{ctn}$, and secondary antibodies coupled to 5- and 10-nm gold particles, respectively. All samples were viewed in a JEOL JEM-1210 transmission electron microscope at 80 kV.

Subcellular Fractionation

To obtain total cell lysates, equal amounts of FosER cells in a 10-cm culture dish or on a 75-mm filter insert were lysed in 1 ml hot SDS sample buffer. For subcellular fractionation in detergent-containing buffers, cells were lysed in 500 μl ice-cold 50 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 0.1 mM DTT, 0.5% Triton X-100, 0.5 mg/ml DNase, 0.2 mg/ml RNase (Boehringer Mannheim, Corp.), and protease inhibitors (1 mM PMSF, 1 mM benzamidine, 10 μg/ml pepstatin, leupeptin, and aprotinin) and incubated for 10 min at 20°C. Triton X-100 was added to a final concentration of 1% and soluble and insoluble fractions were separated by centrifugation at 20,000 rpm for 20 min at 4°C and dissolved in SDS-PAGE sample buffer.

For fractionation in the absence of detergent, FosER cells were scraped off the filters and incubated in 1 ml hypotonic buffer (5 mM Hepes, pH 7.4, 2 mM $MgCl_2$, and 1 mM EGTA) for 15 min on ice. After adjustment to isotonic conditions by addition of 10× buffer A (end concentration, 55 mM Hepes, pH 7.0, 7 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, 0.1 mM DTT, and protease inhibitors), cells were lysed mechanically in a metal ball cell-cracker (EMBL) and total cell lysates were centrifuged at 30,000 *g* in a TLA45 rotor (Beckman) for 20 min at 4°C to separate soluble and insoluble fractions.

Immunoprecipitation

For coimmunoprecipitation analyses, 0.5–1 ml of detergent-free or 1% Triton X-100 containing soluble cell fractions were mixed with 5 μg antibodies or unspecific mouse immunoglobulins as control, and rotated end over end for 2 h at 4°C. After addition of protein G–Sepharose beads and a further incubation for 1 h, the beads were collected by centrifugation and suspended in SDS-PAGE sample buffer.

Other Procedures

SDS-PAGE was performed according to Laemmli (1970). Electrotransfer of proteins onto nitrocellulose (0.2 μm; Schleicher & Schueller) was done in 40 mM glycine, 48 mM Tris, using the BioRad Mini Trans-blot system. For the immunological detection of the proteins, the Protoblot Immunoscreening System (Promega) or the Super Signal ECL system (Pierce Chemical Co.) were used. Quantification of stained protein bands on immunoblots was done using the NIH image software and values were normalized for actin levels in the same samples.

For Northern blot analysis, total RNA was prepared from polarized epithelial cells, 4-d estrogen-treated, and mesenchymal cells (Chomczynski and Sacchi, 1987), transferred to nylon membranes (Hybond N+; Nycomed Amersham, Inc.) and probed for β-catenin mRNA and 28S rRNA using the AlkPhos direct labeling kit (Nycomed Amersham, Inc.) according to the manufacturer's instructions. The EcoRI–BamHI fragment of the β-catenin expression vector pCS2+mtMMBC6*myc (1,276 bp) was used as probe for β-catenin, and the oligo 5′-CCCGCCGGGCTCCCCGGG-3′ for 28S rRNA.

Results

Formation of Junctional Complexes in Polarized FosER Cells

FosER cells formed a tight and well-differentiated epithelial monolayer and developed a high transepithelial electrical resistance (800–1,000 Ohm/cm^2) during the cultivation on porous filter supports in the absence of estradiol for five to eight days (Reichmann et al., 1992). By immunofluorescence and laser scanning confocal microscopy, we investigated the localization of junctional complexes in horizontal and vertical optical sections through these monolayers. E-cadherin, the transmembrane protein of the adherens junctions, and its cytoplasmic interaction partners, β-catenin and $p120^{ctn}$, were exclusively detected at sites of cell–cell contacts at the lateral plasma membrane, clearly visible in vertical optical sections through the epithelial cell monolayer (Fig. 1 A, and data not shown). EM confirmed the formation of a tightly packed epithelial monolayer showing a close alignment of the lateral plasma membranes of adjacent cells with numerous electron-dense cell–cell contact

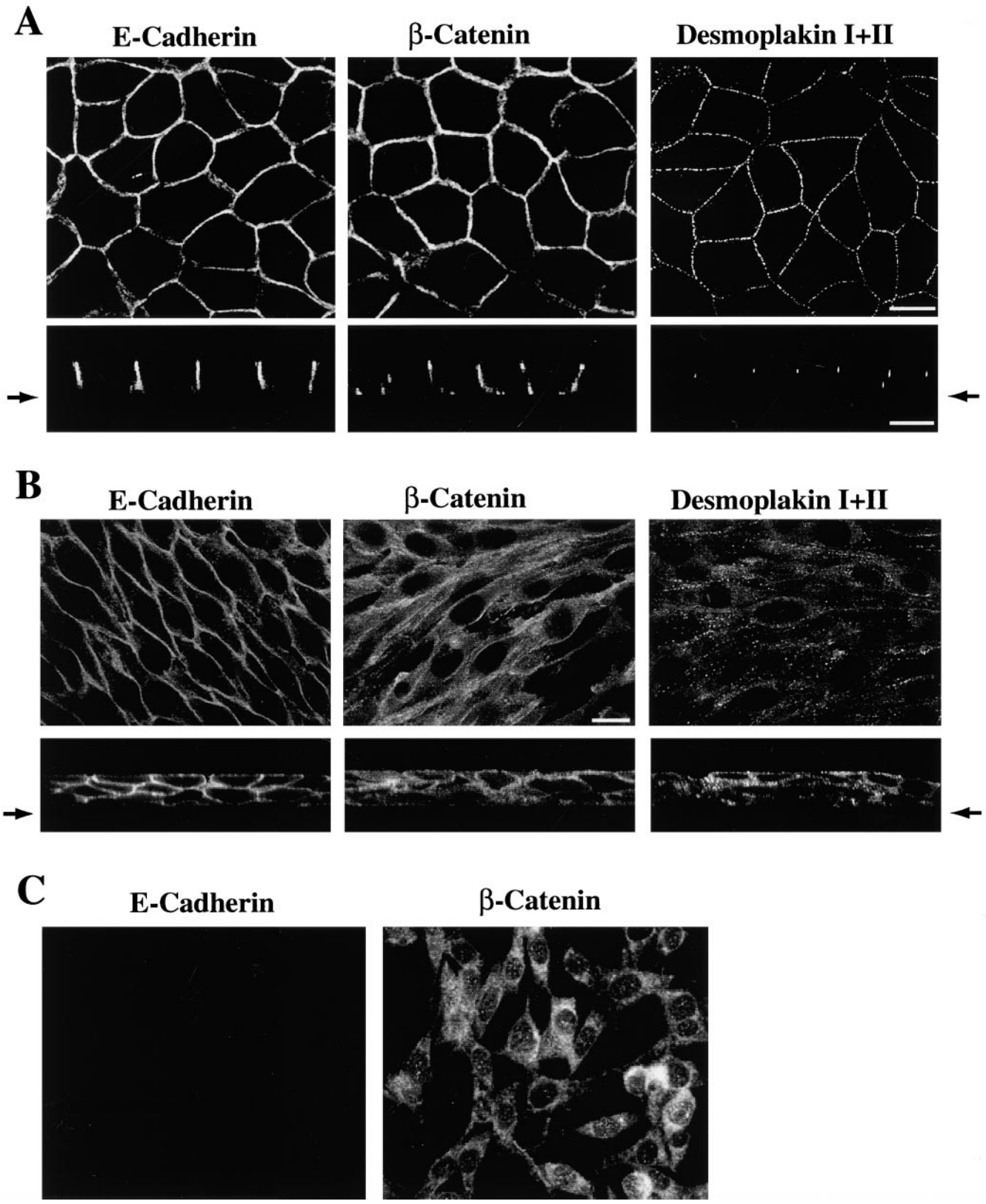

Figure 1. Immunolocalization of junctional proteins in polarized epithelial cell sheets of FosER cells and redistribution upon estradiol treatment. Polarized FosER cells grown on filters in the absence of estradiol (A), cells treated with estradiol for 4 d (B), or mesenchymal FosER cells obtained after estradiol treatment for 14 d (C) were processed for immunofluorescence microscopy using antibodies to junctional proteins as indicated. Confocal images of horizontal (large panels) and vertical (small panels) optical sections through cell layers are shown. Arrows next to vertical sections indicate position of filter. Bars, 10 μm.

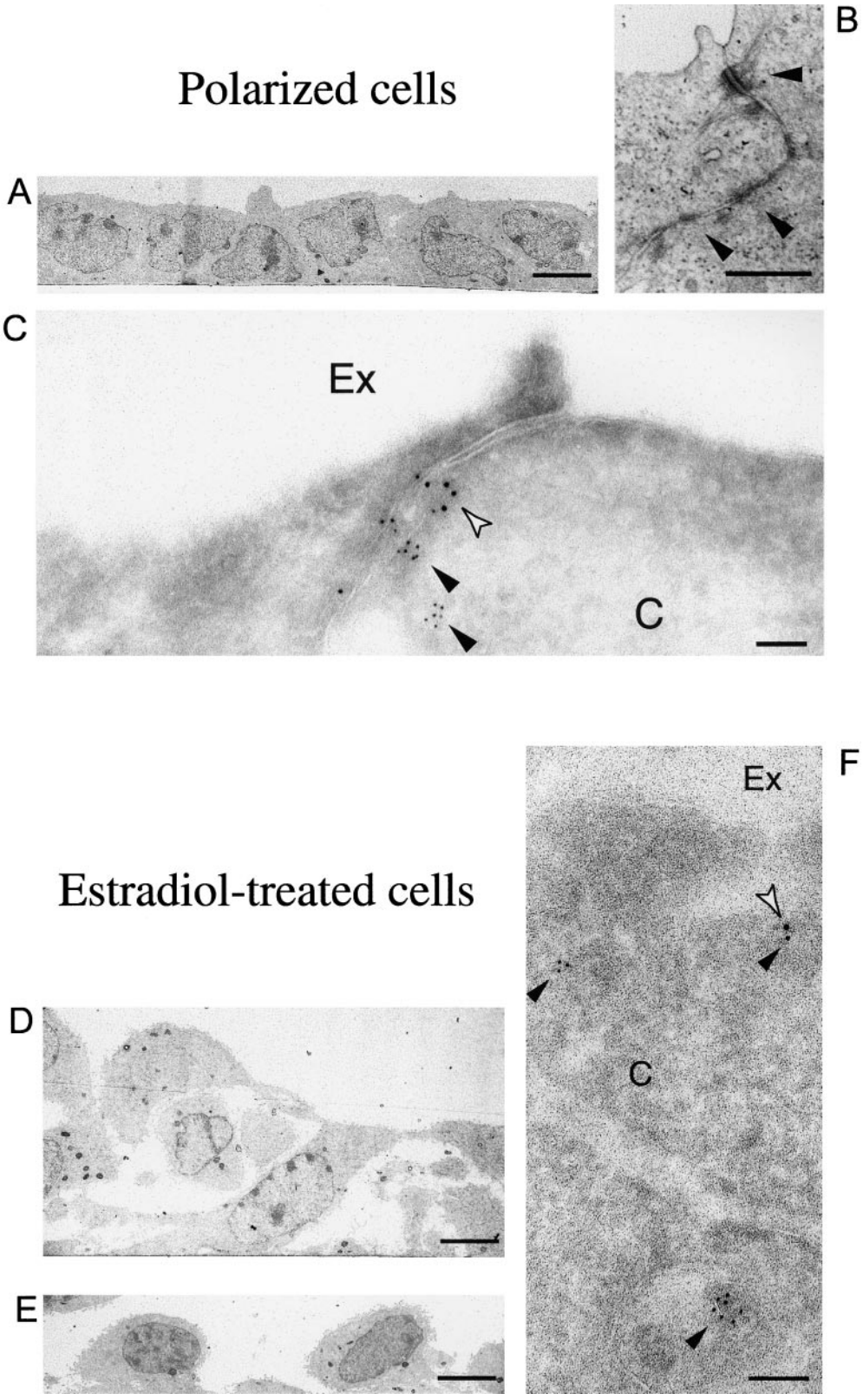

Figure 2. Ultrastructural analysis of polarized FosER cells (A–C) and estradiol-treated cells (D–F). Untreated polarized FosER cells (A–C), FosER cells treated with estradiol for 4 d (D), or mesenchymal cells after 14-d estradiol treatment (E and F) were fixed, embedded in epoxy resins, and ultrathin sections were analyzed by transmission EM (A, B, D, and E); or cells were frozen in liquid nitrogen and cryosections were processed for immunoelectron microscopy (C and F) using antibodies to β-catenin (filled arrowheads) and $p120^{ctn}$ (open arrowheads). C, Cytoplasm; Ex, extracellular space. Bars: (A, D, and E) 10 μm; (B) 500 nm; (C and F) 100 nm.

sites (Fig. 2, A and B, arrowheads). Furthermore, immunogold-labeling of frozen sections of these epithelial cell sheets revealed that β-catenin and $p120^{ctn}$ were closely associated with the lateral membrane mostly concentrated in areas of high electron density, whereas they were absent within the cytoplasm (Fig. 2 C, arrowheads). In addition, the localization of the desmosomal protein desmoplakin in distinct, spot-like structures at more basal regions of the lateral plasma membrane (Fig. 1 A) indicated the existence of desmosomes in these cells. Taken together, these data confirmed and extended earlier findings (Reichmann et al., 1992) showing that FosER cells formed highly polarized

epithelial cell sheets in the absence of estradiol and indicated that β-catenin and p120^{ctn} were predominantly involved in cell–cell adhesion at the lateral plasma membrane in these cells.

Rearrangements of Junctional Proteins upon Loss of Epithelial Polarity and EMT

Next, we studied the fate of junctional complexes and the reorganization of junctional proteins upon addition of estradiol to the culture medium. Within two to four days, cell–cell interactions were significantly reduced, as revealed by EM. Large intercellular gaps formed between membranes of adjacent cells and only a few remnants of cell–cell contact sites were detectable (Fig. 2 D). In addition, cells started to grow in multilayers and the most basal cells in these multilayers dramatically changed their morphology from a cobblestone-like to an extremely elongated spindle-like cell shape (Fig. 1 B).

Confocal immunofluorescence microscopy revealed the disintegration of adherens junctions and desmosomes in the majority of the cells after two to four days of estradiol treatment. In these cells, E-cadherin showed a nonpolar distribution over the entire cell surface (Fig. 1 B). Concomitantly, the localization of the catenins was also markedly altered. A large fraction of β-catenin (Fig. 1 B) and p120^{ctn} (data not shown) colocalized with E-cadherin along the entire surface of the plasma membrane, but a second pool of these catenins was found diffusely distributed throughout the cytoplasm and appeared to be independent of E-cadherin. In addition, desmoplakin disappeared from the plasma membrane and was detected in intracellular distinct dot-like structures (Fig. 1 B), indicating that desmosomal structures were internalized as large complexes upon loss of epithelial polarity.

Mesenchymal cells, obtained by prolonged treatment with estradiol for two weeks or by cultivation after a 48-h pulse of estradiol (Reichmann et al., 1992), exhibited a highly elongated and spindle-shaped phenotype detectable in confocal horizontal sections (Fig. 1 C) and failed to form extensive cell–cell contacts, as seen more clearly in electron micrographs (Fig. 2 E). Immunofluorescence-staining revealed a uniform cytoplasmic distribution of β-catenin (Fig. 1 C) and p120^{ctn} (not shown), as well as intranuclear β-catenin structures. Immunogold EM confirmed that β-catenin and p120^{ctn} were barely detectable at the plasma membrane of mesenchymal cells, but were scattered throughout the cytoplasm (Fig. 2 F, arrowheads) and within the nucleus (see also Fig. 5). In contrast, other junctional proteins, like E-cadherin (Fig. 1 C) and desmoplakin (data not shown) were not detectable above background levels in the mesenchymal FosER cells. Thus, β-catenin and p120^{ctn} were apparently expressed at significant levels in the mesenchymal cells obtained after EMT, but they were mainly found in the cytoplasm and the nucleus, as described previously for β-catenin in a variety of mesenchymal cells (Kim et al., 1998).

Changes in the Expression Levels of Junctional Proteins after EMT

To gain insight in the regulation of junctional proteins after FosER activation, we investigated the expression levels of these proteins at various stages of epithelial depolarization and after FosER-induced EMT. Total cell lysates adjusted to the same total protein content were analyzed by immunoblotting. Strikingly, the overall amounts of junctional proteins did not significantly change during the first four days of estradiol treatment, except for a slight, transient upregulation of desmoplakin I + II (Fig. 3 A). In the mesenchymal cells obtained after EMT, the expression profile of junctional proteins was markedly altered. In accordance with the immunofluorescence studies, the expression of the epithelial-specific proteins, E-cadherin and desmoplakin I +II, was almost completely downregulated (Fig. 3 A, mes). In contrast, the mesenchymal cells retained 10–30% of the β-catenin level present in the epi-

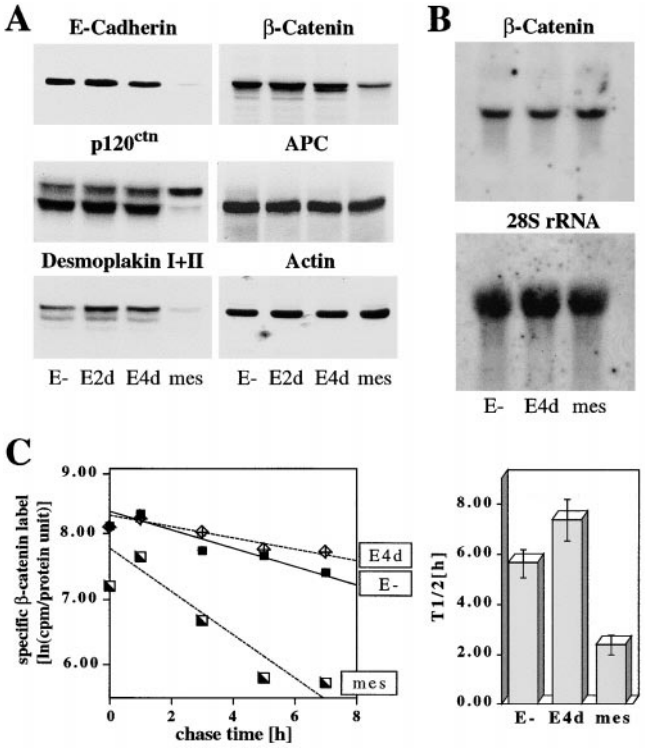

Figure 3. Protein expression levels of junctional proteins (A), and β-catenin mRNA levels (B) and β-catenin turnover rates (C) during various stages of estradiol treatment. A, Equivalent amounts of total cell lysates of untreated polarized FosER cells (E−), cells treated with estradiol for 2 d (E2d) and 4 d (E4d), and mesenchymal FosER cells after 14-d estradiol treatment (mes) were analyzed by immunoblotting using antibodies to indicated proteins. B, Northern blot analysis of total RNA from these cells probed with β-catenin cDNA fragment and with 28S rRNA oligo as a control for equal loading. C, Cells were metabolically labeled with [^{35}S]methionine and chased in unlabeled medium for 1–7 h. β-Catenin was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and autoradiography. The specific β-catenin label was determined by counting radioactivity in the β-catenin band in a scintillation counter. Values were normalized for protein content, determined by densitometric scanning of Coomassie stained gel bands, and plotted against the chase time in a semilogarithmic graph. Best linear curve fits were made from values between 2- and 7-h chase time (one particular experiment is shown), and half-lives (T1/2) were calculated from triplicate analyses (bar graph). Mean values and SD were: E−, 5.64 h, SD = 0.56; E4d, 7.35 h, SD = 0.84; and mes, 2.37 h, SD = 0.38.

thelial FosER cells before estradiol treatment. Control blots of the same lysates with antibodies against actin confirmed equal loading. Interestingly, we observed a dramatic change in the expression pattern of p120^{ctn} proteins. In the epithelial FosER cells, p120^{ctn} was detected as a closely spaced double-band of ~100 kD and a weaker double-band at ~120 kD (Fig. 3 A). The ~100-kD proteins were strongly downregulated in the mesenchymal cells, whereas the ~120-kD proteins persisted and became the predominant p120^{ctn} forms after EMT. It is likely that these different forms of p120^{ctn} represent isoforms of the protein derived by the usage of alternative start codons and by alternative splicing of COOH-terminal exons. Although the specific isoforms expressed in epithelial and mesenchymal FosER cells remain to be identified, our findings correlate with the previously observed predominant expression of 100- and 120-kD p120^{ctn} isoforms in epithelial and in highly motile fibroblastoid cells, respectively (Reynolds and Daniel, 1997).

To test whether the decrease in β-catenin level, which we observed upon FosER-induced EMT, resulted from changes in the expression of the β-catenin gene, as previously reported for E-cadherin (Reichmann et al., 1992), or from a change in the turnover rate of the protein, we performed Northern blot analysis and pulse–chase metabolic labeling experiments. Northern blot analysis using total RNA of epithelial FosER cells, of four-day estradiol-treated, and of mesenchymal cells revealed that the amount of β-catenin mRNA was similar in all cell types (Fig. 3 B). In the pulse–chase experiments, epithelial, four-day estradiol-treated, and mesenchymal FosER cells were pulse-labeled for 90 min using [^{35}S]methionine and chased in unlabeled medium for up to seven hours. From the decrease in the specific β-catenin label, we calculated a half-life of five to six hours for β-catenin in epithelial cells (Fig. 3 C). Whereas the β-catenin half-life was slightly increased to six to eight hours upon four-day estradiol treatment, it significantly dropped to values below 2.6 h in mesenchymal FosER cells. Thus, it is very likely that an increased turnover rate of β-catenin in mesenchymal cells versus epithelial cells, which is likely caused by the downregulation of E-cadherin, leads to a reduction in total β-catenin protein level. Nevertheless, the half-life of β-catenin in mesenchymal cells was still significantly higher than those reported for the protein in various E-cadherin–deficient cell lines (below 40 min; see Papkoff, 1997; Rubinfeld et al., 1997), and is close to values observed upon stabilization of β-catenin by wnt (Papkoff et al., 1996) and by proteosomal protease inhibitors (Aberle et al., 1997), or close to the half-life of stabilized mutated β-catenin in melanoma cells (Rubinfeld et al., 1997). These data indicated that, compared with other E-cadherin–lacking cells, in mesenchymal FosER cells, β-catenin seems to be stabilized to a certain extent also by an E-cadherin–independent mechanism.

Complex Stability and Interactions of Junctional Proteins upon Epithelial Depolarization and EMT

Our data showed significantly reduced cell–cell adhesion at the initial stages of estradiol-treatment, which was completely lost after prolonged treatment. The loss of cell adhesion is usually correlated with a disintegration of all adhesive junctions at the lateral membrane. Therefore, we wanted to investigate if and how the interactions between junctional proteins were changed during this process. To specifically analyze the potential dissociation of β-catenin from the E-cadherin complex, we performed subcellular fractionation of cell lysates made in the absence of Triton X-100. As expected, the membrane-anchored E-cadherin was exclusively found in the pellet fraction upon centrifugation of the lysate at 30,000 *g* (Fig. 4 A). Although the majority of β-catenin was also found in the pellet fraction of epithelial and four-day estradiol-treated cells, a minute amount of soluble β-catenin found in polarized cells was strongly increased upon estradiol-treatment of the cells for four days (Fig. 4 A). This increase of cytoplasmic-free β-catenin was even more evident after immunoprecipitating the protein from the detergent-free supernatant fractions (Fig. 4 B, −TX 100). Immunoblot analysis of the immunoprecipitates using an antibody to E-cadherin confirmed the absence of detectable amounts of E-cadherin. Thus, an E-cadherin–independent pool of β-catenin clearly increased during FosER-induced depolarization of the cells. To test whether this E-cadherin–independent pool of β-catenin interacted with APC, as previously reported for various colon carcinoma cell lines (Barth et al., 1997), we probed the β-catenin immunoprecipitates with antiserum to APC. Although APC was clearly present in polarized, as well as unpolarized and mesenchymal FosER cells (Fig. 3 A), we did not observe a significant coprecipitation of APC with β-catenin at either stage.

Interestingly, in the detergent-free lysates of mesenchymal cells, the majority of β-catenin persisted in the insoluble fraction (Fig. 4 A, mes). As these cells lack E-cadherin, which could recruit β-catenin to the insoluble membrane fraction, other cytoskeletal associations and/or the nuclear translocation of β-catenin might be responsible for its insolubility in the mesenchymal cells.

To analyze potential changes in the stoichiometry of β-catenin and E-cadherin in β-catenin complexes after FosER-induced epithelial depolarization and EMT, we lysed the cells in buffers containing 1% Triton X-100, which solubilized a significant fraction of adherens junction proteins (data not shown). Immunoprecipitates obtained with β-catenin antibodies contained similar amounts of β-catenin and E-cadherin in both polarized and unpolarized cells (Fig. 4 B, + TX-100). None of these proteins were brought down in control immunoprecipitates with irrelevant antibodies (data not shown). As there were no significant changes in the stoichiometry of the proteins in the precipitated complexes within 4-d estradiol treatment, we concluded that the major pool of β-catenin remained associated with E-cadherin in the FosER cells after initial loss of epithelial polarity. The mesenchymal cells after EMT completely lacked E-cadherin, and concomitantly, β-catenin immunoprecipitates did not contain any E-cadherin. Interestingly, these immunoprecipitates did not contain significant amounts of APC (Fig. 4 B), suggesting that the majority of β-catenin in mesenchymal cells existed in APC-independent structures.

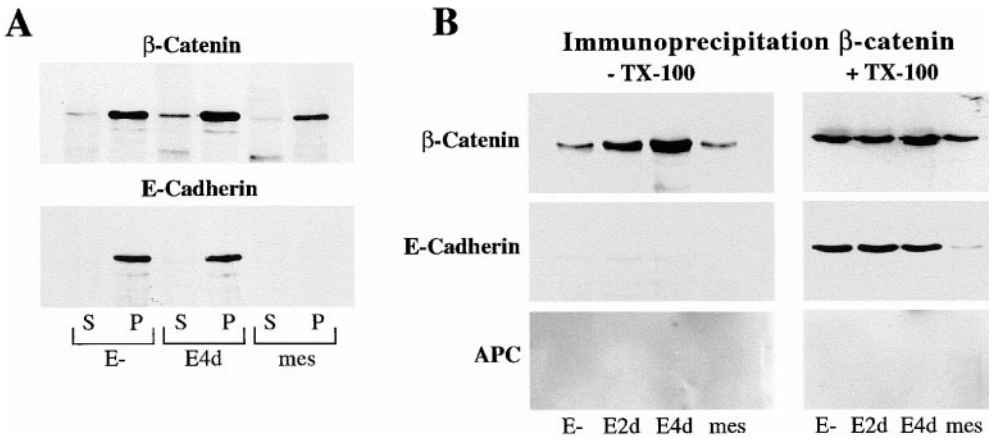

Figure 4. Analysis of the E-cadherin–independent pool of β-catenin and of β-catenin complexes in polarized and estradiol-treated FosER cells. A, Untreated FosER cells (E−), and cells treated with estradiol for 2 d (E2d), and 4 d (E4d), and mesenchymal FosER cells after 14-d estradiol treatment (mes) were lysed in detergent-free buffer and soluble (S) and insoluble (P) cell fractions were obtained by centrifugation of total cell lysates at 30,000 *g* for 20 min. B, Cells were lysed in buffers without (−TX-100) or with (+TX-100) Triton X-100 and β-catenin was immunoprecipitated from the soluble cell fractions. Immunoblots of cell fractions and of β-catenin immunoprecipitates using antibodies to indicated proteins are shown.

Nuclear Translocation of β-Catenin and p120^{ctn} in Mesenchymal FosER Cells

To further analyze the subcellular distribution of β-catenin in the absence of endogenous E-cadherin, we studied the mesenchymal cells after EMT by confocal laser scanning microscopy. Confocal images of horizontal optical sections through mesenchymal cells revealed a mostly diffuse staining of β-catenin in the cytoplasm (Fig. 1 C). In the majority of cells, however, β-catenin was also detected in intranuclear structures (Fig. 1 C). At higher magnification, brightly stained, discrete spot-like β-catenin structures were visible throughout the nucleus (Fig. 5 A, whole-mount). Costaining of nuclei with the DNA stain propidium iodide confirmed that the optical sections were put through the middle of the nucleus and that the β-catenin structures were indeed intranuclear. As the nuclear staining of β-catenin might be underrepresented due to a restricted accessibility of the antibodies to the β-catenin protein within the bulky chromatin structure in whole-mount preparations, we also performed cryosectioning of frozen cells. Immunofluorescence microscopy of these sections strongly labeled dot-like β-catenin structures in the nuclear compartment (Fig. 5 A, cryosection). In addition, we performed immunoelectron microscopy of frozen sections to confirm our immunofluorescence data on the ultrastructural level and to perform semiquantitative statistical analysis. In these sections, nuclei were clearly discernable due to the electron-dense chromatin structures and the bilayered nuclear envelope (Fig. 5 B). At higher magnification, β-catenin–specific immunogold label was clearly detectable in clusters both within the nucleus (Fig. 5 B, N) and in the cytoplasm (Fig. 5 B, C) of mesenchymal cells (Fig. 5 B, arrowheads). A crude quantification of the intranuclear versus cytoplasmic distribution of β-catenin was obtained by counting the number of gold particles in the nucleus and in the surrounding cytoplasm in comparable areas on the sections. According to this quantification, there was a significant translocation and accumulation of β-catenin in the nucleus upon FosER-induced EMT (Fig. 5 C).

Interestingly, immunoelectron microscopy demonstrated that p120^{ctn} protein was also detectable in nuclei of mesenchymal FosER cells, although to a lesser extent than β-catenin (Fig. 5 C). These data show for the first time that p120^{ctn} resembles β-catenin in its ability to translocate to the nucleus. Interestingly, p120^{ctn} has recently been demonstrated by yeast two-hybrid and coimmunoprecipitation studies to interact with Kaiso, a member of the POZ-ZF transcription factor family, which was localized in intranuclear dot-like structures (Daniel and Reynolds, 1999).

Interaction of β-Catenin with a LEF-1–related Protein in Mesenchymal FosER Cells

The nuclear localization of β-catenin in mesenchymal FosER cells prompted us to test for interactions of β-catenin with LEF-1/TCF transcription factors. Immunoblot analysis of total cell lysates, using an affinity-purified LEF-1 antibody (Huber et al., 1996b), revealed a major band at ~50 kD and several minor bands, suggesting that LEF-1 and potential LEF-1/TCF isoforms are expressed in mesenchymal FosER cells (Fig. 6 A, cell lysate). Furthermore, the antibody detected brightly stained spot-like structures scattered throughout the nucleus in both polarized and mesenchymal FosER cells (Fig. 6 B). Double immunofluorescence microscopy using antibodies to β-catenin and LEF-1 showed that the LEF-1–specific staining colocalized frequently with that of nuclear β-catenin in mesenchymal cells, whereas in polarized cells, β-catenin was restricted to the plasma membrane and did not overlap with the nuclear LEF-1 structures (Fig. 6 B). Thus, unlike polarized cells, in mesenchymal cells LEF-1 and β-catenin may form complexes within the nucleus. To investigate on the biochemical level whether β-catenin interacted with LEF-1–related proteins, we lysed mesenchymal FosER cells in buffers containing Triton X-100 and DNase and performed coimmunoprecipitation analysis from the soluble fraction using β-catenin antibodies. Both β-catenin and the LEF-1–related protein of ~50 kD were detected in the immunoprecipitate by immunoblotting, whereas they were absent in control precipitates using an unrelated mouse antibody (Fig. 6 A).

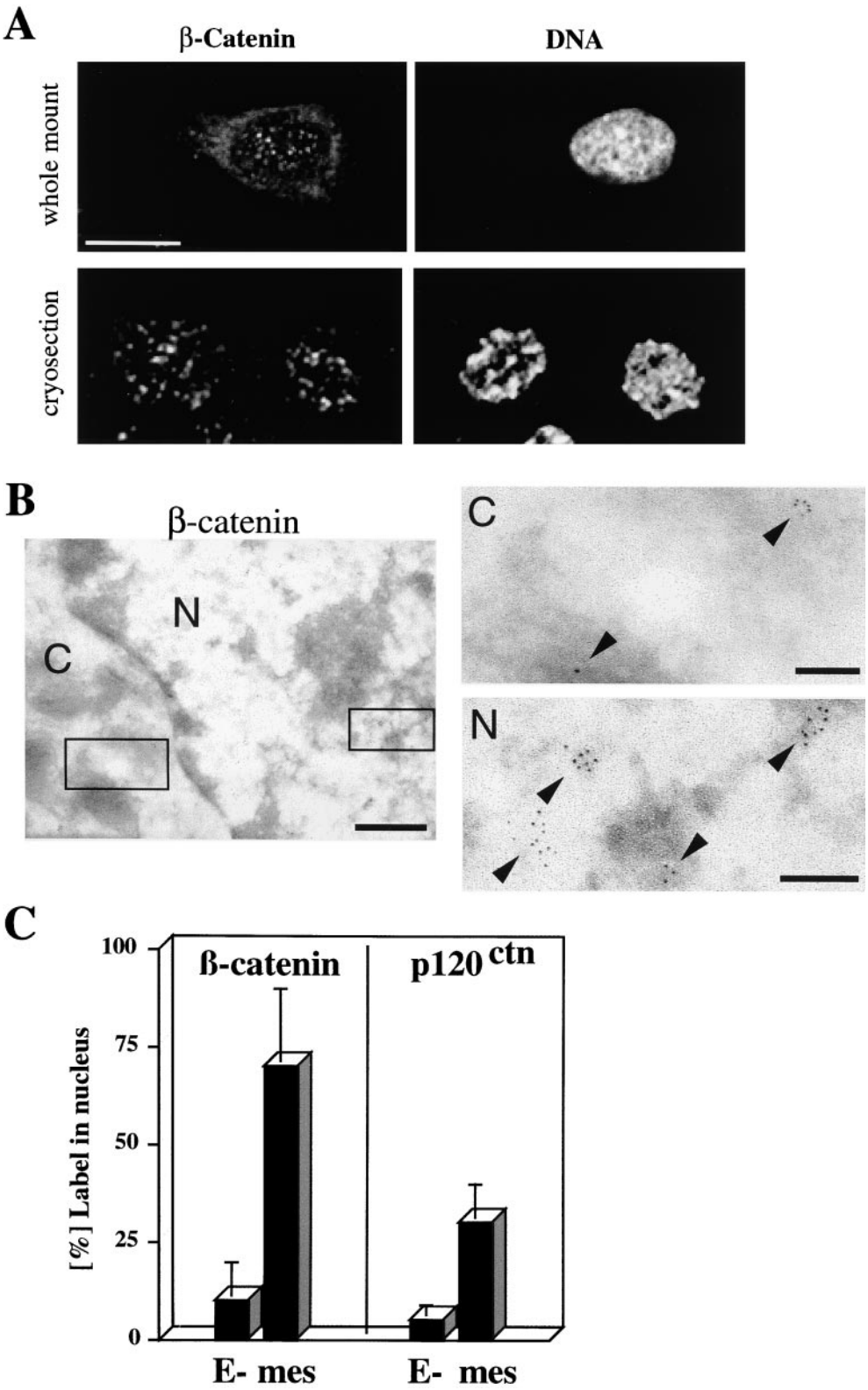

Figure 5. Nuclear localization of β-catenin and p120^{ctn} after EMT. A, Mesenchymal FosER cells after 14-d estradiol treatment (whole-mount) or cryosections obtained from these cells were processed for immunofluorescence microscopy using antibodies to β-catenin and the DNA stain propidium iodide. Double fluorescence confocal images are shown. Bar, 10 μm. B, Cryosections of mesenchymal FosER cells were processed for immunoelectron microscopy using antibodies to β-catenin. Low magnifications (left) and higher magnifications (right) of indicated areas are shown. Arrowheads denote gold label. C, cytoplasm, N, nucleus. Bars: (left) 500 nm; (right) 100 nm. C, Semiquantitative analyses of the relative amount of β-catenin- and p120^{ctn}-specific label in the nucleus versus cytoplasm in polarized epithelial (E−) and mesenchymal (mes) FosER cells. The number of gold particles was counted in equivalent areas of the cytoplasm and the nucleus and the percentage of nuclear particles was calculated. Data represent statistical mean values of 50 counted samples. Mean values and SD were: β-catenin, E− (10%, SD = 10), mes (70%, SD = 20); and p120^{ctn}, E− (5% SD = 4), mes (30% SD = 10).

Activation of FosER Augments β-Catenin/LEF-1–dependent Transcriptional Activation

To test the functional significance of the nuclear translocation of β-catenin and its association with nuclear LEF-1 upon EMT, we performed a reporter gene assay using the pTopflash/pFopflash reporter constructs, previously shown to specifically reveal LEF-1/TCF/β-catenin–dependent transcriptional activity (Korinek et al., 1997). Polarized FosER cells, four-day estradiol-treated, and mesen-

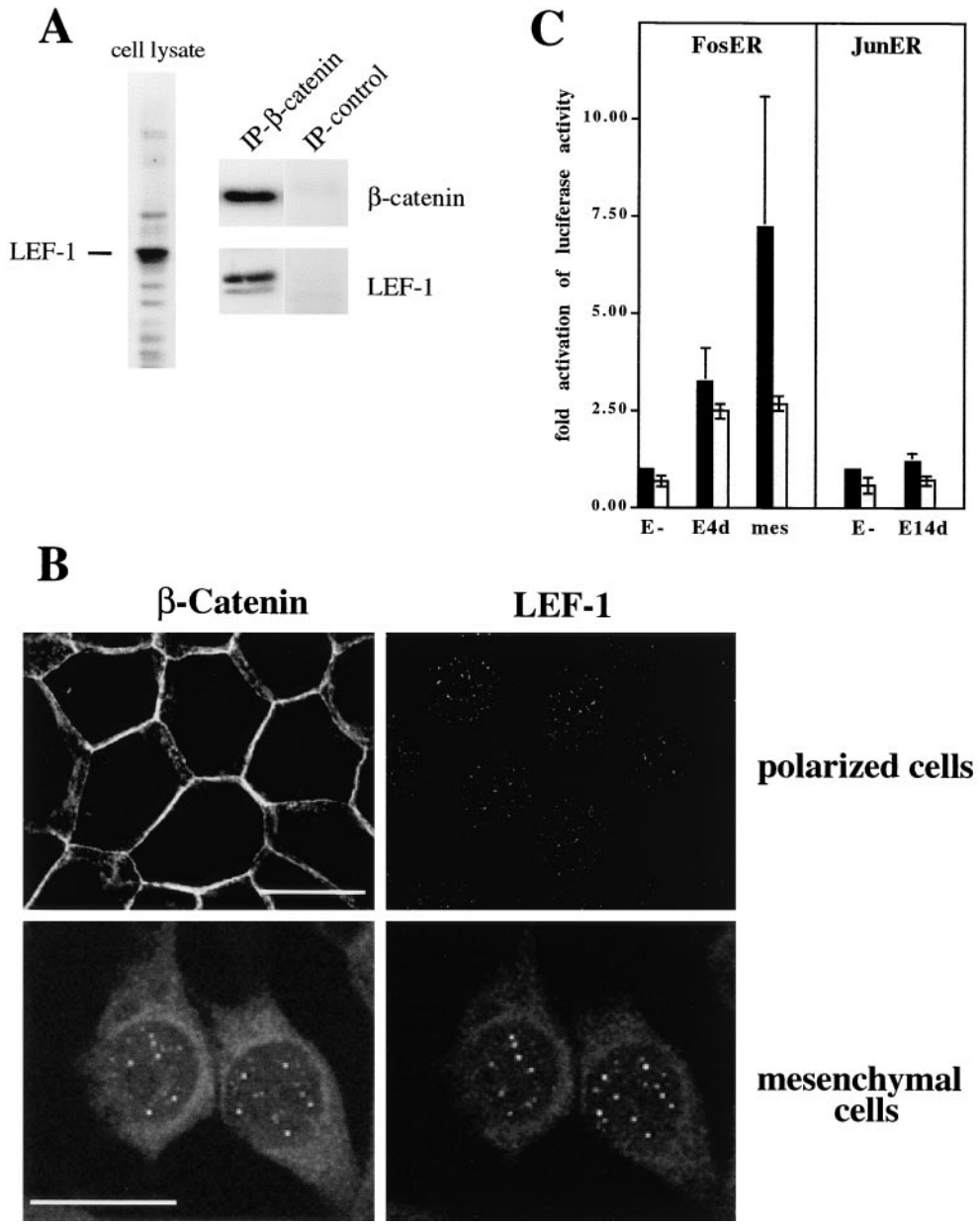

Figure 6. Formation of a transcriptionally active β-catenin/LEF-1 complex in mesenchymal FosER cells after 14-d estrogen treatment. A, Mesenchymal FosER cells were lysed in buffer containing Triton X-100 and DNase, and β-catenin was immunoprecipitated from the soluble cell fraction. Control immunoprecipitates were obtained with an unrelated mouse IgG. Immunoblots of the total cell lysate using affinity-purified antiserum to LEF-1 and relevant areas of immunoblots of the β-catenin (IP-β-catenin) and control (IP-control) immunoprecipitates using antibodies to β-catenin and LEF-1 are shown. B, Polarized or mesenchymal FosER cells were treated for immunofluorescence microscopy using an mAb to β-catenin and an affinity-purified antibody to LEF-1. Confocal double immunofluorescence images are shown. Bar, 10 μm. C, Polarized epithelial (E−), 4-d estradiol-treated (E4d) and mesenchymal (mes) FosER cells after 14-d estradiol treatment, or polarized (E−) and 14-d estradiol-treated JunER cells were transiently transfected with TOPFLASH luciferase reporter plasmid containing multimerized LEF-1/TCF binding sites (filled bars) or mutated FOPFLASH plasmid (open bars). The level of luciferase activity was normalized for the expression of a cotransfected CMV–β-galactosidase expression plasmid. Fold activation was quantitated relative to the level of luciferase activity from polarized FosER and JunER cells, respectively. All experiments were performed three to five times. Mean values and SD were: FosER cells/TOPFLASH, E− (1.0); E4d (3.28, SD = 0.87); mes (7.23, SD = 3.36); FosER cells/FOPFLASH, E− (0.7, SD = 0.15); E4d (2.5, SD = 0.20); mes (2.7, SD = 0.20); JunER cells/TOPFLASH cells, E− (1.0); E14d (1.23, SD = 0.18); and JunER cells/FOPFLASH, E− (0.60, SD = 0.2); E14d (0.73, SD = 0.13).

chymal cells were transiently transfected with the reporter plasmid, together with a CMV promoter-driven β-galactosidase reporter construct to normalize for transfection efficiency. Normalized luciferase reporter activity was already slightly elevated in unpolarized FosER cells and was further increased in mesenchymal cells up to a maximum, tenfold higher than the basal activity seen in control epithelial cells (Fig. 6 C, filled bars). Using the pFopflash reporter construct containing a mutant version of the LEF/TCF binding sites, we also observed a slight increase of the luciferase activity upon four-day estradiol treatment, but the activity was not further upregulated in mesenchymal cells (open bars). As the specific stimulation of LEF-1/TCF transcriptional activity seen with the pTopflash reporter construct strictly depends on the formation of a complex of LEF/TCF factors with β-catenin (Korinek et al., 1997; Hsu et al., 1998), we concluded that β-catenin formed a transcriptionally active complex with LEF-1/TCF-related proteins in the nucleus of mesenchymal cells.

To address the question whether induction of LEF-1/TCF transcriptional activity is specific for EMT or just a consequence of increased AP-1 activity, the same assay was performed with the related mammary cell line JunER, which stably expresses a c-JunER fusion protein. JunER cells showed a dramatic loss of epithelial polarity upon incubation with estradiol, but completely failed to undergo

EMT even after prolonged estradiol treatment (Fialka et al., 1996). Upon transfection of the reporter constructs into JunER cells, no significant increase of luciferase reporter activity relative to the basal activity in polarized cells was detected upon estradiol activation of the JunER cells for up to 14 d (Fig. 6 C). JunER cells do not lose E-cadherin, but show a similar upregulation of AP-1 target genes (Fialka et al., 1996) as Fos-ER cells. Therefore, we conclude that the increase of LEF-1/TCF-dependent transcriptional activity detected in FosER cells upon estradiol treatment was not solely caused by estrogen-induced AP-1 activation.

To provide additional support for the idea that E-cadherin–independent β-catenin is directly responsible for the increase of LEF-1/TCF transcriptional activity upon FosER-induced EMT, we transiently transfected an E-cadherin expression construct into epithelial or mesenchymal FosER cells, together with the LEF-1/TCF reporter plasmids. Ectopically expressed E-cadherin in E-cadherin–negative embryonic stem cells and in SW480, NIH3T3, and 293-T cells (Orsulic et al., 1999), or even transfected cytoplasmic domains of N- or E-cadherin and derivatives thereof containing the β-catenin binding site (Sadot et al., 1998), recently have been shown to compete with endogenous LEF-1/TCF proteins for binding to β-catenin and, hence, reduced the transcriptional activation of the reporter gene. Cotransfection of the E-cadherin expression vector in two- to tenfold excess over the LEF-1/TCF reporter construct into mesenchymal FosER cells caused a progressive reduction of reporter luciferase activity (12% decrease at twofold excess to 30–50% reduction at a tenfold excess; Fig. 7, filled bars). Expression of exogenous E-cadherin in mesenchymal cells was confirmed by immunoblotting, revealing a significant increase of the E-cadherin signal in the transfected cells (Fig. 7). In epithelial FosER cells, the same amounts of exogenous E-cadherin had less pronounced effects on the already low, basal level of transcriptional activity (Fig. 7, open bars).

If the reexpression of E-cadherin caused a reduction of LEF-1/TCF-dependent transcriptional activity by recruiting soluble β-catenin to the plasma membrane, exogenous

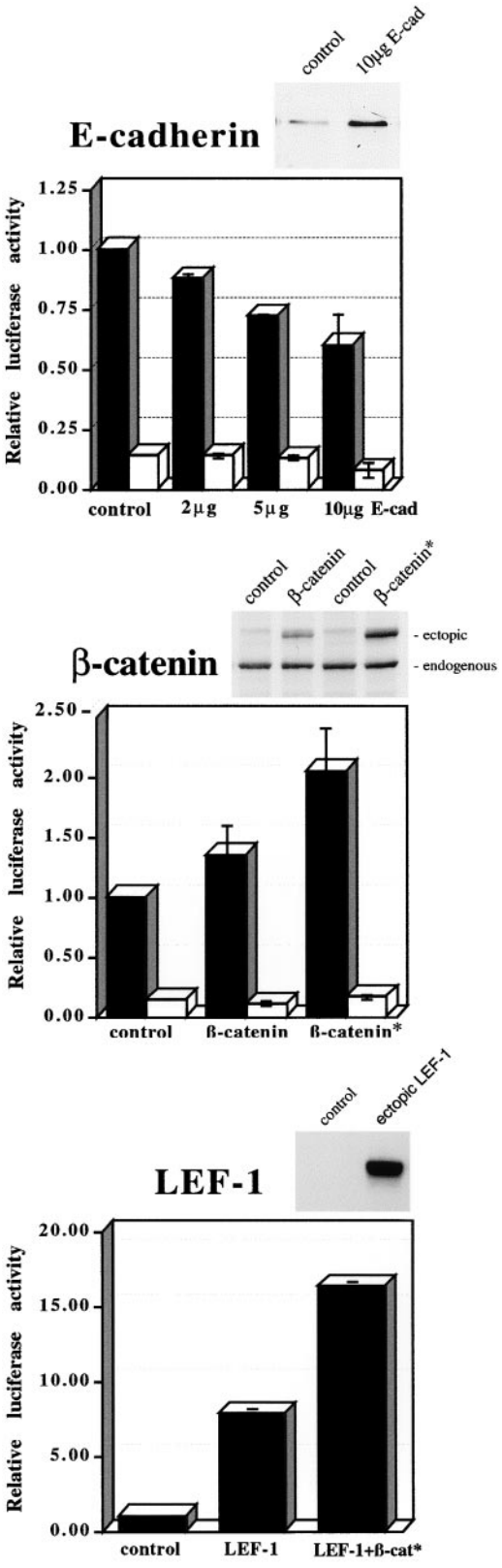

Figure 7. Dependence of β-catenin/LEF-1 transcriptional activity on ectopic expression of E-cadherin, wild-type and mutated β-catenin, and LEF-1. Mesenchymal (filled bars) and polarized epithelial (open bars) FosER cells were transiently transfected with TOPFLASH luciferase reporter plasmid, together with increasing amounts of expression plasmids (2–10 μg) encoding E-cadherin or expression plasmids encoding myc-tagged β-catenin, stable, mutated β-catenin*, or expression plasmids encoding HA-tagged LEF-1 alone or in combination with mutated β-catenin (β-cat*). The level of luciferase activity was normalized for the expression of a cotransfected CMV–β-galactosidase expression plasmid. Luciferase activities relative to controls obtained by cotransfections with empty expression vectors are shown. Experiments were repeated at least twice for each E-cadherin expression study and at least three times for all others. Means and SD were: for E-cad/mes, 2 μg (0.88, SD = 0.02); 5 μg (0.72, SD = 0.01); 10 μg (0.60, SD = 0.13); for Ecad/pol, 2 μg (0.14, SD = 0.01); 5 μg (0.13, SD = 0.01); 10 μg (0.08, SD = 0.03); for βcat/mes (1.35, SD = 0.25); βcat*/mes (2.05, SD = 0.35); βcat/pol (0.11, SD = 0.02); βcat* (0.16, SD = 0.02); for LEF-1 (7.85, SD = 0.35); and LEF+βcat* (16.35, SD = 0.35). Experiments were also performed once with mutated FOPFLASH plasmid, showing no significant effect upon ectopic protein expression. The expression of ectopic proteins is shown by immunoblot analyses of total cell lysates of transfected mesenchymal cells using antibodies to E-cadherin, β-catenin, and the HA tag.

expression of β-catenin should enhance transcriptional activation by LEF-1/TCF. Indeed, cotransfection of reporter constructs with expression plasmids for myc-tagged, wild-type β-catenin, or NH_2 terminally mutated, nondegradable β-catenin (Aberle et al., 1997) further increased the high luciferase activity in mesenchymal cells (Fig. 7, filled bars). While transfection of full-length β-catenin further enhanced the already elevated reporter gene expression in mesenchymal cells ~1.4-fold over mock transfected samples, mutated stable β-catenin augmented luciferase activity more than twofold. Ectopic expression of myc-tagged β-catenin was verified by immunoblotting (Fig. 7). The expression of β-catenin in polarized FosER cells had only minor effects on the reporter gene activity (Fig. 7, open bars). Taken together, the transfection studies point to an elevated β-catenin/LEF-1 transcriptional activity in mesenchymal versus polarized cells, which is apparently dependent on the amount of soluble, E-cadherin–independent β-catenin.

To test whether the high β-catenin–dependent transcriptional activity in mesenchymal cells is also dependent on the amount of LEF-1 present in the cells, we cotransfected a mouse LEF-1 expression plasmid together with the reporter construct into the cells. Transcriptional activity in cells expressing HA-tagged exogenous LEF-1 (Fig. 7, see immunoblot) was eightfold higher than the activity in mock transfected cells, and could be further elevated twofold upon coexpression of mutated exogenous stable β-catenin (16-fold over mock transfected cells; Fig. 7).

Our results clearly demonstrated that endogenous β-catenin and endogenous LEF-1/TCF proteins in mesenchymal FosER cells were able to form a transcriptionally active complex, which could be increased further upon ectopic expression of β-catenin and LEF-1 without additional signals.

Discussion

EMT, the conversion of epithelial cells into a fibroblastoid, mesenchymal phenotype occurs both during early embryonic development and during progression of cancer to metastatic stages (Oft et al., 1996, 1998). EMT seems to involve a complex series of overlapping signaling pathways and molecular mechanisms, whose interdependence is still poorly understood. The previously established FosER cell line represents an ideal and unique cell culture model system to study the regulation of the emerging dual role of E-cadherin and catenins in cell adhesion and signaling at various stages of epithelial depolarization and after EMT, since depolarization and EMT can be conditionally induced by estradiol-dependent induction of FosER protein function. Our studies revealed that depolarization and EMT involved clearly discernable steps, which may include different molecular mechanisms. Initially, cells lost epithelial polarity, as indicated by a redistribution of E-cadherin–containing complexes over the entire plasma membrane. In a second process, a small cytoplasmic pool of β-catenin accumulated, which did not interact with E-cadherin. This β-catenin pool apparently also failed to associate with the β-catenin downregulating APC complex. The third important, but clearly delayed, event in FosER-induced EMT was the complete loss of E-cadherin protein, which has been shown to be correlated with the upregulation of vimentin and other mesenchymal markers (Reichmann et al., 1992). The complete loss of E-cadherin in the mesenchymal state is likely to further increase the free pool of β-catenin and to make it increasingly available for signaling processes. The fourth mechanism involved the accumulation of β-catenin in the nucleus, its interaction with LEF-1–related proteins, and the activation of LEF-1/β-catenin–dependent transcriptional activity. Compared with previously published experiments on the cell adhesion and signaling activities of β-catenin in mammalian cell systems, our observations provide novel insights into these processes for the following reasons. First, most previous studies on cell adhesion and/or signaling functions of β-catenin in mammalian cells used either transformed cell lines, which expressed abnormally high levels of β-catenin (Korinek et al., 1997; Rubinfeld et al., 1997; Hart et al., 1998), or highly overexpressed β-catenin or β-catenin–binding proteins in a normal β-catenin background (Behrens et al., 1996, 1998; Huber et al., 1996b; Ikeda et al., 1998; Simcha et al., 1998). In contrast to these studies, we present evidence for the involvement of endogenous β-catenin expressed at physiological levels in both cell adhesion and signaling. Therefore, potential artifacts produced by the high unphysiological level of these proteins can be mostly excluded in our studies. Second, we show and compare endogenous β-catenin activities in a single cell line at various cellular phenotypes, i.e., in the polarized stage, upon depolarization, and upon EMT.

Interactions of β-Catenin with E-Cadherin Are Predominant and May Regulate β-Catenin Functions

Our experiments showed that E-cadherin is the predominant interaction partner of endogenous β-catenin. Although the disintegration of junctional complexes and the redistribution of junctional proteins was already observed after two days of estradiol treatment, the majority of β-catenin remained associated with E-cadherin. However, the small fraction of E-cadherin–independent β-catenin in the cytoplasm found after two days of estrogen treatment was clearly increased after four-day treatment, and was consistent with the diffuse cytoplasmic staining of β-catenin found in immunofluorescence images at this stage. As we already found a slight increase in β-catenin/LEF-1 transcriptional activity after four-day estrogen treatment, this cytoplasmic pool of β-catenin might be involved in signaling. A large amount of E-cadherin–independent β-catenin, however, was only found in the mesenchymal cells, when E-cadherin was no longer expressed at significant levels. As β-catenin/LEF-1 transcriptional activity was clearly upregulated in the mesenchymal cells, it may be concluded that E-cadherin downregulation is important for the regulation of β-catenin signaling activity in FosER cells. This hypothesis is further confirmed by our observation that a related cell line (JunER) expressing a conditionally active JunER-fusion protein lost epithelial polarity upon induction of JunER activity (Fialka et al., 1996), but did not lose E-cadherin expression nor upregulate β-catenin/LEF-1 transcriptional activity.

The notion that the expression level of E-cadherin influences β-catenin/LEF-1 transcriptional activity is also

supported by the expression of exogenous E-cadherin in mesenchymal cells, which led to a clear reduction of LEF-1–dependent luciferase activity. Although alternative mechanisms cannot be ruled out completely, it is most likely that ectopically expressed E-cadherin bound β-catenin and caused a reduction in the free pool of monomeric β-catenin available for signaling. These results are in accordance with previous studies, showing a negative effect of E-cadherin expression on the signaling activity of β-catenin during early developmental stages of *Xenopus laevis* (Heasman et al., 1994; Fagotto et al., 1996). Furthermore, it has recently been shown that transient expression of full-length E-cadherin or of its cytoplasmic, β-catenin–binding domain into various mammalian cell lines induced the formation of complexes of β-catenin with ectopic proteins at the plasma membrane or in the nucleus and inhibited β-catenin/LEF-1–mediated transactivation (Sadot et al., 1998; Orsulic et al., 1999). In our studies, the observed ~30–50% downregulation of β-catenin/LEF-1 transactivation activity upon transient E-cadherin expression in mesenchymal FosER cells was slightly less than the 50–70% decrease reported upon expression of E- or N-cadherin in E-cadherin–negative embryonic stem cells or in SW480 cells (Sadot et al., 1998; Orsulic et al., 1999). This difference could be explained by different expression levels of ectopic E-cadherin in these cell systems.

It is still unclear how the expression of endogenous E-cadherin is regulated during EMT. In vivo footprinting analyses revealed that positive regulatory elements of the E-cadherin promoter were specifically occupied by transcription factors in polarized, but not in mesenchymal, FosER cells (Hennig et al., 1995). Moreover, a LEF-1/TCF recognition site has been identified upstream of these elements, which was shown to bind to a β-catenin/LEF-1 complex in vitro (Huber et al., 1996b). It remains to be determined whether β-catenin/LEF-1 complexes recognize this site also in vivo and whether they influence the expression of E-cadherin in a positive or negative fashion. Furthermore, β-catenin protein levels may also indirectly influence the expression level of E-cadherin by stabilizing E-cadherin in junctional complexes.

β-Catenin Signaling Is Upregulated in Mesenchymal FosER Cells

Considering the criteria currently applied to identify β-catenin as a signaling component in a given cellular system, our data clearly indicated that β-catenin fulfills signaling functions upon FosER-induced EMT. We found that E-cadherin–independent β-catenin in mesenchymal FosER cells was not associated with APC. As APC and axin have been shown previously to downregulate β-catenin in colon carcinoma cells (Rubinfeld et al., 1997; Behrens et al., 1998; Hart et al., 1998), the lack of an APC/β-catenin complex in FosER cells might cause the stabilization of cytoplasmic β-catenin and make it available for its function in transcriptional activation. In line with this, previous reports implicated only uncomplexed monomeric β-catenin in signaling functions, while β-catenin linked to various cytoplasmic complexes did not participate in signaling (Papkoff et al., 1996; Stewart and Nelson, 1997). In addition, our pulse–chase experiments indicated that the stability of β-catenin in mesenchymal cells is higher than that reported in other E-cadherin–deficient cells and was close to that of apparently transcriptionally active β-catenin in melanoma cells or in wnt-induced cells (Papkoff et al., 1996; Rubinfeld et al., 1997).

β-Catenin was also detected in the nucleus of mesenchymal FosER cells. Many previous studies showed a strong and more or less diffuse nuclear staining of β-catenin upon gross overexpression of β-catenin and/or LEF-1 in mammalian cells (Behrens et al., 1996; Huber et al., 1996b). In contrast, our studies identified endogenous β-catenin in small spot-like structures within the nucleus. As many of these structures were also stained by an affinity-purified anti–LEF-1 antibody, they likely represent functional β-catenin/LEF-1 complexes. Interestingly, ectopic expression of β-catenin in MDCK cells also revealed a concentration of both β-catenin and LEF-1 in distinct nuclear structures, rather than a diffuse nuclear staining of these proteins (Simcha et al., 1998).

The notion that transcriptionally active β-catenin/LEF-1 complexes were formed in estradiol-treated FosER cells was supported by our observation that LEF-1/TCF–dependent luciferase reporter activity was clearly elevated in the mesenchymal cells. The level of transcriptional β-catenin/LEF-1 activation after FosER-induced EMT was clearly in the range of that reported in previous studies upon expression of β-catenin and/or LEF-1 and/or wnt in various cell systems (Korinek et al., 1997; Porfiri et al., 1997; Hsu et al., 1998), or upon activation of integrin-linked kinase (Novak et al., 1998), and can, therefore, be considered as significantly high to be of functional relevance. Furthermore, in earlier observations, relatively small differences in free β-catenin on the dorsal versus ventral side of the *Xenopus* embryo resulted in dorsal mesoderm formation (Larabell et al., 1997), indicating that even subtle changes in the level of signaling competent β-catenin may have a drastic impact on gene activation.

Potential Molecular Mechanisms Leading to the Formation of Active β-Catenin/LEF-TCF Complexes in FosER Cells

The molecular mechanisms underlying the formation of an active β-catenin/LEF-TCF complex in mesenchymal FosER cells remain unclear. Activation of the FosER fusion protein might trigger specific signaling pathways that regulate the interaction of β-catenin with the transcription factors. Potential candidates for these additional signals have been reported in various cell systems. In NIH3T3 cells, ectopically expressed wnt/wingless, a regulator of GSK3β activity during embryonic development, was described to induce the formation of a transcriptionally active LEF-1/β-catenin complex in cells overexpressing LEF-1, whereas LEF-1 expression alone was not sufficient (Hsu et al., 1998). Similarly, wnt-1 expression in PC12 and 293 cells (Porfiri et al., 1997), and in a mammary epithelial cell line (Korinek et al., 1998; Papkoff and Aikawa, 1998) has been shown to induce the formation of active LEF-1/β-catenin and TCF4/β-catenin complexes, respectively. Furthermore, overexpression of integrin-linked kinase (ILK) in mammary epithelial cells caused translocation of β-catenin into the nucleus and induction of LEF/TCF

transcriptional activity (Novak et al., 1998). Thus, activation of FosER and integrin linked kinase might trigger signaling pathways that are linked to the wnt-pathway or regulate the formation of an active nuclear β-catenin/LEF-1 complex via as yet unknown mechanisms.

In summary, our data showed that upon FosER-induced EMT of mammary epithelial cells, β-catenin/LEF-1 transcriptional activity is activated. Future studies involving forced expression of E-cadherin in these cells at various stages of EMT and the identification of β-catenin/LEF-1 target genes will help to identify the contributions of β-catenin signaling to EMT and to unravel the specific functions of β-catenin.

We wish to thank Stefan Grünert, IMP Vienna, for valuable comments on the manuscript. Furthermore, we thank Rolf Kemler and Otmar Huber, Max Planck Institute for Immunobiology, Freiburg, Germany for providing β-catenin expression plasmids and LEF-1 antisera; Gerhard Christofori, IMP Vienna, for the E-cadherin expression vector; Hans Clevers, University Hospital, Utrecht, The Netherlands, for the TCF-dependent luciferase reporter constructs; Inke Näthke, University of Dundee, Scotland, for antiserum to APC; Ernst Müllner, Department of Molecular Biology, University of Vienna, for the 28S rRNA probe; and D. Gründler-Thompson, Boehringer-Ingelheim, Vienna for the β-galactosidase reporter plasmid.

This study was supported by grants from the Austrian Science Research Fund (FWF) No. SFB 006 to R. Foisner and H. Beug.

Submitted: 10 May 1999
Revised: 3 December 1999
Accepted: 6 December 1999

References

Aberle, H., H. Schwartz, and R. Kemler. 1996. Cadherin–catenin complex: protein interactions and their implications for cadherin function. *J. Cell Biochem*. 61:514–523.

Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler. 1997. Beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO (Eur. Mol. Biol. Organ) J.* 16:3797–3804.

Adams, C.L., Y.T. Chen, S.J. Smith, and W.J. Nelson. 1998. Mechanisms of epithelial cell–cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin–green fluorescent protein. *J. Cell Biol.* 142:1105–1119.

Barth, A.I., I.S. Nathke, and W.J. Nelson. 1997. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. *Curr. Opin. Cell Biol.* 9:683–690.

Behrens, J., B.A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich, and W. Birchmeier. 1998. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science.* 280:596–599.

Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature.* 382:638–642.

Ben-Ze'ev, A., and B. Geiger. 1998. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol.* 10:629–639.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. 162:156–159.

Cowin, P., and B. Burke. 1996. Cytoskeleton-membrane interactions. *Curr. Opin. Cell Biol.* 8:56–65.

Daniel, J.M., and A.B. Reynolds. 1997. Tyrosine phosphorylation and cadherin/catenin function. *BioEssays.* 19:883–891.

Daniel, J.M., and A.B. Reynolds. 1999. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol. Cell. Biol.* 19:3614–3623.

de La Coste, A., B. Romagnolo, P. Billuart, C.A. Renard, M.A. Buendia, O. Soubrane, M. Fabre, J. Chelly, C. Beldjord, A. Kahn, and C. Perret. 1998. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA*. 95:8847–8851.

Eger, A., and R. Foisner. 2000. Dynamics and cross-talk of junctional proteins: a molecular basis for the regulation of cell adhesion and epithelial polarity. *Protoplasma.* In press.

Eger, A., A. Stockinger, G. Wiche, and R. Foisner. 1997. Polarisation-dependent association of plectin with desmoplakin and the lateral submembrane skeleton in MDCK cells. *J. Cell Sci.* 110:1307–1316.

Fagotto, F., N. Funayama, U. Gluck, and B.M. Gumbiner. 1996. Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in *Xenopus. J. Cell Biol.* 132:1105–1114.

Fialka, I., H. Schwarz, E. Reichmann, M. Oft, M. Busslinger, and H. Beug. 1996. The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. *J. Cell Biol.* 132:1115–1132.

Gat, U., R. DasGupta, L. Degenstein, and E. Fuchs. 1998. De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell*. 95:605–614.

Giese, K., and R. Grosschedl. 1993. LEF-1 contains an activation domain that stimulates transcription only in a specific context of factor-binding sites. *EMBO (Eur. Mol. Biol. Organ) J.* 12:4667–4676.

Gumbiner, B.M. 1997. Carcinogenesis: a balance between beta-catenin and APC. *Curr. Biol.* 7:R443–R446.

Hart, M.J., R. de los Santos, I.N. Albert, B. Rubinfeld, and P. Polakis. 1998. Downregulation of beta-catenin by human axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr. Biol.* 8:573–581.

He, T.C., A.B. Sparks, C. Rago, H. Hermeking, L. Zawel, L.T. da Costa, P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. *Science*. 281:1509–1512.

Heasman, J., A. Crawford, K. Goldstone, P. Garner-Hamrick, B. Gumbiner, P. McCrea, C. Kintner, C.Y. Noro, and C. Wylie. 1994. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell*. 79:791–803.

Hennig, G., J. Behrens, M. Truss, S. Frisch, E. Reichmann, and W. Birchmeier. 1995. Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. *Oncogene*. 11:475–484.

Hinck, L., I.S. Nathke, J. Papkoff, and W.J. Nelson. 1994. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* 125:1327–1340.

Hsu, S.C., J. Galceran, and R. Grosschedl. 1998. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. *Mol. Cell. Biol.* 18:4807–4818.

Huber, O., C. Bierkamp, and R. Kemler. 1996a. Cadherins and catenins in development. *Curr. Opin. Cell Biol.* 8:685–691.

Huber, O., R. Korn, J. McLaughlin, M. Ohsugi, B.G. Herrmann, and R. Kemler. 1996b. Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* 59:3–10.

Ikeda, S., S. Kishida, H. Yamamoto, H. Murai, S. Koyama, and A. Kikuchi. 1998. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta–dependent phosphorylation of beta-catenin. *EMBO (Eur. Mol. Biol. Organ) J.* 17:1371–1384.

Itoh, K., V.E. Krupnik, and S.Y. Sokol. 1998. Axis determination in *Xenopus* involves biochemical interactions of axin, glycogen synthase kinase 3 and beta-catenin. *Curr. Biol.* 8:591–594.

Kim, K., K.J. Daniels, and E.D. Hay. 1998. Tissue-specific expression of β-catenin in normal mesenchyme and uveal melanomas and its effect on invasiveness. *Exp. Cell Res.* 245:79–90.

Korinek, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a beta-catenin–Tcf complex in $APC^{-/-}$ colon carcinoma. *Science.* 275: 1784–1787.

Korinek, V., N. Barker, K. Willert, M. Molenaar, J. Roose, G. Wagenaar, M. Markman, W. Lamers, O. Destree, and H. Clevers. 1998. Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* 18:1248–1256.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Larabell, C.A., M. Torres, B.A. Rowning, C. Yost, J.R. Miller, M. Wu, D. Kimelman, and R.T. Moon. 1997. Establishment of the dorso–ventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* 136:1123–1136.

Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers. 1996. XTcf-3 transcription factor mediates beta-catenin–induced axis formation in *Xenopus* embryos. *Cell.* 86:391–399.

Moon, R.T., J.D. Brown, and M. Torres. 1997. WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* 13:157–162.

Morin, P.J., A.B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K.W. Kinzler. 1997. Activation of beta-catenin–Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science.* 275:1787–1790.

Nagafuchi, A., S. Ishihara, and S. Tsukita. 1994. The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin–α-catenin fusion molecules. *J. Cell Biol.* 127:235–245.

Nagar, B., M. Overduin, M. Ikura, and J.M. Rini. 1996. Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature.* 380:360–364.

Nathke, I.S., L. Hinck, J.R. Swedlow, J. Papkoff, and W.J. Nelson. 1994. Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. *J. Cell Biol.* 125:1341–1352.

Novak, A., S.C. Hsu, C. Leung-Hagesteijn, G. Radeva, J. Papkoff, R. Montesano, C. Roskelley, R. Grosschedl, and S. Dedhar. 1998. Cell adhesion and

the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. USA*. 95:4374–4379.

Oft, M., J. Peli, C. Rudaz, H. Schwarz, H. Beug, and E. Reichmann. 1996. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev*. 10:2462–2477.

Oft, M., K.H. Heider, and H. Beug. 1998. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr. Biol.* 8:1243–1252.

Orford, K., C. Crockett, J.P. Jensen, A.M. Weissman, and S.W. Byers. 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. *J. Biol. Chem*. 272:24735–24738.

Orsulic, S., O. Huber, H. Aberle, S. Arnold, and R. Kemler. 1999. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1–mediated transactivation. *J. Cell Sci.* 112:1237–1245.

Ozawa, M., and R. Kemler. 1998. The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. *J. Cell Biol.* 142:1605–1613.

Papkoff, J. 1997. Regulation of complexed and free catenin pools by distinct mechanisms. *J. Cell Biol.* 272:4536–4543.

Papkoff, J., and M. Aikawa. 1998. WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. *Biochem. Biophys. Res. Commun*. 247:851–858.

Papkoff, J., B. Rubinfeld, B. Schryver, and P. Polakis. 1996. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell. Biol.* 16:2128–2134.

Perl, A.K., P. Wilgenbus, U. Dahl, H. Semb, and G. Christofori. 1998. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature.* 392:190–193.

Porfiri, E., B. Rubinfeld, I. Albert, K. Hovanes, M. Waterman, and P. Polakis. 1997. Induction of a beta-catenin–LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. *Oncogene*. 15:2833–2839.

Reichmann, E., H. Schwarz, E.M. Deiner, I. Leitner, M. Eilers, J. Berger, M. Busslinger, and H. Beug. 1992. Activation of an inducible c-FosER fusion protein causes loss of epithelial polarity and triggers epithelial–fibroblastoid cell conversion. *Cell.* 71:1103–1116.

Reynolds, A.B., and J.M. Daniel. 1997. p120ctn: a Src-substrate turned catenin. *In* Cytoskeletal-Membrane Interactions and Signal Transduction. P. Cowin and M.W. Klymkowsky, editors. Springer-Verlag, Heidelberg, Germany. 31–48.

Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu, and P. Polakis. 1996. Binding of GSK3beta to the APC–beta-catenin complex and regulation of complex assembly. *Science*. 272:1023–1026.

Rubinfeld, B., P. Robbins, M. El-Gamil, I. Albert, E. Porfiri, and P. Polakis. 1997. Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science*. 275:1790–1792.

Sadot, E., I. Simcha, M. Shtutman, A. Ben-Ze'ev, and B. Geiger. 1998. Inhibition of β-catenin–mediated transactivation by cadherin derivatives. *Proc. Natl. Acad. Sci. USA*. 95:15339–15344.

Shapiro, L., A.M. Fannon, P.D. Kwong, A. Thompson, M.S. Lehmann, G. Grubel, J.F. Legrand, J. Als-Nielsen, D.R. Colman, and W.A. Hendrickson. 1995. Structural basis of cell–cell adhesion by cadherins. *Nature.* 374:327–337.

Simcha, I., M. Shtutman, D. Salomon, J. Zhurinsky, E. Sadot, B. Geiger, and A. Ben-Ze'ev. 1998. Differential nuclear translocation and transactivation potential of beta-catenin and plakoglobin. *J. Cell Biol.* 141:1433–1448.

Stewart, D.B., and W.J. Nelson. 1997. Identification of four distinct pools of catenins in mammalian cells and transformation-dependent changes in catenin distributions among these pools. *J. Biol. Chem*. 272:29652–29662.

Tomschy, A., C. Fauser, R. Landwehr, and J. Engel. 1996. Homophilic adhesion of E-cadherin occurs by a co-operative two-step interaction of N-terminal domains. *EMBO (Eur. Mol. Biol. Organ) J.* 15:3507–3514.

Weiss, E.E., M. Kroemker, A.H. Rudiger, B.M. Jockusch, and M. Rudiger. 1998. Vinculin is part of the cadherin–catenin junctional complex: complex formation between alpha-catenin and vinculin. *J. Cell Biol.* 141:755–764.

Willert, K., and R. Nusse. 1998. Beta-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev*. 8:95–102.

Yap, A.S., W.M. Brieher, M. Pruschy, and B.M. Gumbiner. 1997. Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function. *Curr. Biol.* 7:308–315.

Yap, A.S., C.M. Niessen, and B.M. Gumbiner. 1998. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J. Cell Biol.* 141:779–789.

Yost, C., M. Torres, J.R. Miller, E. Huang, D. Kimelman, and R.T. Moon. 1996. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev*. 10:1443–1454.